The alpha-7 nicotinic acetylcholine receptor is involved in a direct inhibitory effect of nicotine on GnRH release: in vitro studies

Running title: Effect of nicotine on GnRH neurons

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Abstract

The activation of nicotinic cholinergic receptors (nAChR) inhibits the reproductive axis; however, it is not clear whether nicotine may directly modulate the release of hypothalamic gonadotropin-releasing hormone (GnRH). Experiments carried out in GT1-1 immortalized GnRH neurons reveal the presence of a single class of high affinity $\alpha4\beta2$ and $\alpha7$ nAchR subtypes. The exposure of GT1-1 cells to nicotine does not modify the basal accumulation of GnRH. However, nicotine was found to modify GnRH pulsatility in perifusion experiments and inhibits, the release of GnRH induced by prostaglandin E_1 or by K^+-induced cell depolarization; these effects were reversed by D-tubocurarine and α-bungarotoxin. In conclusion, the results reported here indicate that: functional nAChRs are present on GT1-1 cells, the activation of the α-bungarotoxin-sensitive subclass ($\alpha7$) produces an inhibitory effect on the release of GnRH and that the direct action of nicotine on GnRH neurons may be involved in reducing fertility of smokers.

Keywords

GnRH, nicotine, prostaglandin E1, neurons, reproduction

1. Introduction

Cigarette smoking may have adverse effects on fertility. It has been shown that the activation of nicotinic acetylcholine receptors (nAChR) inhibits the activity of the hypothalamus-pituitary-gonadal axis at several levels. For example, it has been reported that nicotine inhibits steroidogenesis in Leydig cells as well as sperm motility and oocyte maturation (Condorelli et al., 2013; Gocze et al., 1996; Yamamoto et al., 1998; Zenzes, 2000).

In vivo and *in vitro* activation of acetylcholine receptors leads to either stimulatory or inhibitory effects on GnRH secretion (Kalra and Kalra, 1983; Kawai et al., 2013; Koren et al., 1992; Richardson et al., 1982), leaving the nature of action of cholinergic input on GnRH neurons unresolved.

It has also been shown that the specific activation of nAChR affects the hypothalamic-pituitary-gonadal axis. In particular, nicotine induces a decrease of the release of luteinizing hormone possibly acting at the pituitary level (Blake et al., 1972; Fiorindo and Martini, 1975; Kanematsu and Sawyer, 1973; Motta et al., 1973; Zemkova et al., 2013). However, it is not clear whether nicotine can modulate the release of the hypothalamic gonadotropin releasing hormone (GnRH).

It has been shown that nicotine administration may inhibit the activity of the gonadotropin-releasing hormone (GnRH) pulse generator in ovariectomized rats (Sano et al., 1999). However, it has been postulated that nicotine action could be mediated by a potentiation of the inhibitory tone exerted by opioid peptides on GnRH/gonadotropin release (Hodson et al., 1997); conversely, experimental evidence might exclude this hypothesis (Sano et al., 1999). Kimura and coworkers (Kimura et al., 2004) have further reported that, in the cultured embryonic olfactory placode, nicotine inhibits GnRH secretion through a release of GABA and the consequent activation of GABA-A receptor system.

It cannot be excluded that the nicotinic cholinergic system might also act directly on GnRH secreting neurons, as acetylcholine induces a rapid, but transient, stimulation of GnRH release in perifused hypothalamic and immortalized GnRH neurons (Krsmanovic et al., 1998).
Considering that the affinity of nicotine to nAChR ranges from pM to nM values and that after inhalation of a single puff of cigarette smoke, the nicotine concentration in human arterial plasma rise to a peak of about 50-100 ng/ml (0.3-0.6 μM) in about 20 sec (Crandall et al., 1989), an effect of nicotine on GnRH release should be carefully evaluated.

Neuronal nAChRs are a heterogeneous family of acetylcholine (Ach)-gated channels with a pentameric structure resembling that of muscle AchRs. Mammalian nAChRs can be subdivided into two main classes: homomeric or heteromeric α-bungarotoxin (αBgtx)–sensitive receptors consisting of α7, α9, α9-α10, α7β2 subunits, and αBgtx-insensitive heteromeric receptors consisting of α2–α6 and β2–β4 subunits (reviewed in (Millar and Gotti, 2009; Zoli et al., 2015)). The two classes of receptors are characterized by distinct pharmacological profiles wherein: the αBgtx-insensitive heteromeric receptors are bound by nicotine agonists with a very high affinity but not by the antagonist αBgtx, whereas the αBgtx–sensitive receptors are bound by agonists with lower affinity but with high αBgtx affinity (Dutton and Craik, 2001). All of the nAChR subtypes, but in particular the α7 subtype, show pronounced permeability for Ca^{2+} relative to Na^{+}; and many of the biological functions identified for nAChRs have been associated with receptor-mediated changes in intracellular Ca^{2+} concentration which modulate the release of several neurotransmitters. Data from distribution of nAChR in mouse brain indicate that α4β2 and α7 are present in the hypothalamic region (see (Millar and Gotti, 2009) for a review) while α9 is expressed predominantly in hair cells of the cochlea.
In the present work we evaluated the presence of nicotinic receptors and the nature of a direct effect of nicotine on GnRH secretion in a neuronal isolated system using cell lines of immortalized hypothalamic GnRH neurons, an *in vitro* model widely used to study the mechanisms that control GnRH release in controlled conditions (Glidewell-Kenney et al., 2013; Gore and Roberts, 1997; Krsmanovic et al., 1998; Maggi et al., 1995; Maggi et al., 1995; Maggi et al., 2000; Mellon et al., 1990; Pal et al., 2007; Pimpinelli et al., 1999).

2. Materials and Methods

2.1 Chemicals

Nicotine tartrate and prostaglandin E_1 (PGE_1) were from Sigma Chemicals (St.Louis, MO), nonradioactive epibatidine□□ α-bungarotoxin (αBgtx) and D-tubocurarine (D-Tub) were from Tocris Bioscience (Bristol, UK). When not specified, other reagents were from Sigma Chemicals (St.Louis, MO).

2.2 Cell cultures

GT1-1 cells, generously provided by Dr. R.I. Weiner (San Francisco, CA) through Dr. B. Marchetti (Catania, Italy) and human embryonic kidney cells (HEK)293 were routinely grown in monolayer at 37 °C in a humidified CO2 incubator in Dulbecco's Minimum Essential Medium (DMEM) containing 1 mM sodium pyruvate, 100 mg/ml streptomycin, 100 U/ml penicillin and 10 mg/l of phenol red (Biochrom KG, Berlin, Germany) and supplemented with 10% fetal calf serum (FCS, Gibco, Grand Island, NY). The medium was replaced at 2-day intervals. Subconfluent cells were routinely harvested by trypsinization and seeded in 57 cm^2 dishes (1 x 10^6 cells) for propagation. For all the experiments, GT1-1 cells within 6 passages were used.

Receptor binding

Subconfluent GT1-1 or HEK293 cells were detached from the subconfluent culture using phosphate buffer saline (PBS) containing 1 mM PMFS, washed twice by centrifugation, resuspended in 50 mM TrisHCl pH 7.4 containing 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 5 mg/ml mixture of the protease inhibitors leupeptin, bestatin, pepstatin A, aprotinin and 1 mM PMFS and homogenized to obtain a crude membrane preparation.

All the incubations were performed in a buffer containing 50 mM Tris-HCl pH 7, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 2 mg/ml BSA.

The binding of ^{3}H-Epibatidine (Epi, epibatidine specific activity: 66 Ci/mmol was purchased from Perkin Elmer (Waltham, MA, USA), and ^{125}I-α-bungarotoxin (αBgtx, specific activity: 122.8 Ci/mmol was purchased from Perkin Elmer (Waltham, MA, USA) were performed by a homogeneous saturation binding assay according to a mixed protocol combining both saturation of labeled ligand (the first 7-8 concentrations of respectively 0.005-5 nM and 0.01-20 nM) and displacement curves (the last 3-4 concentrations of 50-1000 nM for both ligands) with unlabeled ligand (Rovati et al., 1989). By effectively combining both saturation and competition protocols in a single curve, high ligand concentrations can be reached without using excessive amounts of labeled ligand (the competition part of the curve), while retaining adequate radioactivity in the lower concentration range (the saturation part of the curve).

Non-specific binding (averaging 5-10% of total binding for Epi and 25-30% of total binding for αBgtx was determined in the presence of 2 μM unlabeled ligands.

Saturation experiments were performed by incubating aliquots of GT1-1 crude membrane preparations with ligands overnight at 20°C. At the end of the incubation, the samples were filtered through a 24-channel Brandel cell harvester on GFC filters presoaked in buffer +1% BSA, five times with ice-cold buffer, and the bound radioactivity then determined by means of liquid scintillation spectrometer (Packard 1600 CA, Packard, Milano, Italy) with 60% of efficiency.

2.3 RT-PCR assay

For expression studies, cells were washed with cold PBS, and collected with TRIzol (Invitrogen) and total RNA was extracted following the manufacturer's protocol. Mouse hypothalami, collected from adult animals, were homogenized in TRIzol and RNA extracted following the manufacturer's protocol. One microgram of total RNA was subjected to cDNA synthesis with Superscript II reverse transcriptase (Invitrogen), using random hexamers according to standard procedures. PCRs were

performed using Taq PCR Core Kit (Qiagen) and the following oligonucleotides; (α4) forward 5'-CAATGTACACCACCGCTCAC-3' and reverse 5'-TGGTCTGACACTGGAAGCTG-3', (α7) forward 5'-GCACCTCATGCATGGTACAC-3' and reverse 5'-ATCCAGAGTGGGCAATGAC-3', (α9) forward 5'-CCTTGCGTCCTCATATCGTT-3' and reverse 5'-CCCTGGAAGTTTGCCATAAA-3', (β2) forward 5'-TGGCTGTGTTCAGGGGTTTT-3' and reverse 5'-CCTCAATCTTGCATGCGCTC-3'.
The mouse *Gapdh* gene was analyzed as housekeeping gene with the following PCR primers: forward 5'-GGCCCCTCTGGAAAGCTGTGG-3' and reverse 5'-TCTTGCTCAGTGTCCTTGCTGGG-3'.
Amplification products were separated by 1% agarose gel electrophoresis and detected by ethidium bromide fluorescence on a UV transilluminator (Bio-Rad).

2.4 GnRH release form perifused GT1-1 cells

Perifusion experiments were performed as already described (Magni et al., 1999). In brief, GT1–1 cells were grown on Cytodex-3 beads (Pharmacia Biotech, Uppsala, Sweden). After 3–4 days, cells were loaded into temperature-controlled glass syringes, the final cell-matrix volume was adjusted to 0.15 ml. Chambers were perifused at a flow rate of 10 ml/h with Locke's, gassed with 95% O2–5% CO2 at 37 C. After a 2-h equilibration period, samples were collected every 90 sec and stored at -20 C until radioimmunoassayed for GnRH. Cells were perifused for the first hour with Locke's medium, and then with medium containing nicotine (500 μM) for 30 min. GnRH pulses were identified and their parameters were determined by a computer algorithm cluster analysis (Veldhuis and Johnson, 1986). The occurrence and the duration of pulses are shown above each plot.

2.5 GnRH accumulation in GT1-1 cell culture medium

GT1-1 neurons were plated in 24-well plates (0.5 x 10^6 cells/cm^2) and used after five days of culture. Art the day of the experiment, cells were washed with 1 ml of DMEM (prewarmed at 37° C) and, when not otherwise specified, incubated for 30 min in DMEM containing the substances to be tested. At the end of the incubation period, the medium was collected, centrifuged for 5 min at 12.000 rpm and the supernatant stored at -70 C until the GnRH RIA (Maggi et al., 1995). The cells remaining in the culture wells were collected in 0.2 M NaOH, and assayed for protein content using a microassay with human serum albumin as a standard. No variations of total protein/well were detected in all the experimental groups (data not shown).
For the experiments performed in the presence of depolarizing extracellular concentrations of K^+ ions, DMEM was substituted by Locke's medium containing 5.6 mM or 56 mM K^+ (Pimpinelli et al., 2003).

2.6 GnRH radioimmunoassay

The concentration of GnRH in the media and in the fractions collected during the perifusion as well as static experiments was determined by RIA using a commercial antibody (Cod. L-8391, Sigma Chemicals, St Louis, MO) and iodinated GnRH (Amersham, Milano, Italy). The GnRH standard was from NovaBiochem (Laufelfingen, Switzerland) (Maggi et al., 1995; Pimpinelli et al., 1999). All samples were run in duplicate; the detection limit was 3.9 pg/ml. The inter- and intra-assay coefficients of variation were 9.4% and 6.6%, respectively. Each experiment was repeated at least three times.

2.7 cAMP assay

For cAMP experiments, GT1-1 cells were plated in 24-wells plates (0.5 x 10^6 cells/cm^2) and used after three days of culture (Pimpinelli et al., 1999). All the samples were assayed for protein content using a microassay with human serum albumin as a standard. Intracellular cAMP

accumulation was measured over a 15-minutes incubation period with 1 µM of prostaglandin E1 (PGE_1) (Sigma, St. Louis, MO), as activators of adenylyl cyclase, after a 10 minutes preincubation with 0.5 mM 3-isobutyl-1-methyl-xanthine (Sigma Chemicals, St. Louis, MO). A commercial available binding-protein assay kit (Amersham, Milano, Italy) was used to evaluate cAMP levels in ethanol extracted cells according to manufacturers' instructions.

2.8 Statistical analysis

Receptor binding experiments were optimized with the program DESIGN (Rovati et al., 1990) and the results further analyzed by the program LIGAND (Munson and Rodbard, 1980). The statistic software PRISM was used to analyze the dose-response curves, by a four parameter non-linear regression, and the other results, by ANOVA and adequate post-hoc tests (Dunnett's or Bonferroni's test).

3. Results

3.1 Detection of nAChR in GT1-1 cells

A series of experiments were performed to investigate, using different approaches, the presence of nAChRs in GT1-1 cells.

Receptor binding assays on GT1-1 membrane preparations were carried out using labeled Epi, an azabicycloheptane alkaloid exerting potent nicotinic agonist action for heteromeric receptors, and αBgtx that binds with high affinity the homomeric nAChR. Epi binds to several heteromeric nAChR subtypes with a Kd in the pM range whereas its binding to the α7 homomeric subtype is in the nM range and competitive with that of αBgtx.

We then analyzed the saturation binding of Epi on separate preparations of GT1-1 cell homogenates in the presence of 2 μM unlabeled αBgtx to avoid possible binding to homomeric receptors. In these conditions, a specific and saturable Epi binding with a Kd of 13 pM and a Bmax (mean + SE) of 6.6 + 3.5 fmol/mg of protein was observed (Fig. 1A).

Saturation binding experiments performed with αBgtx, also revealed a single high affinity site with a Kd of 2.8 nM and a Bmax (mean + SE) of 16.6 + 3.5 fmol/mg of protein (Fig. 1B).

As a control the binding of both Epi and αBgtx was tested on membrane preparations from HEK293 cells, that does not express nAchR (Chavez-Noriega et al., 2000; Craig et al., 2004). The results, shown in Fig. 1 A and B clearly indicate a negligible, mainly nonspecific, binding of the two ligands impossible to resolve for parameter estimation by LIGAND program.

These results indicate the presence of independent high affinity binding sites for both Epi and αBgtx on GT1-1 cell membranes, and suggest the presence of at least two distinct populations of nAChR subtypes.

Based on data indicating the presence of $\alpha4\beta2$ and $\alpha7$ nAChR subunits in mouse hypothalamus and transcriptomic analysis (Affymetrics mouse 2.0, data not shown) of GT1 cell extracts revealing the presence of signals for α7, α4 and β2 nicotine receptor subunits, we analyzed the presence of specific transcripts for these nicotine receptor subunits by RT-PCR. The transcript analysis, carried out on total RNA extracted from GT1-1 cells, showed the presence of transcripts for α7, α4 and β2 subunits of nAChR (Fig. 1C). The transcript for α9 subunit, analyzed as a control, was not detectable in GT1-1 cell extract (data not shown).

3.2 Effects of the activation of nAChR by nicotine on basal GnRH release

The effect of receptor activation on the accumulation of GnRH released from GT1-1 cells was evaluated. Graded concentrations of nicotine (1-500 μM) and/or nicotine antagonists (αBgtx and D-Tub) were added to GT1-1 cells maintained in basal culture conditions and GnRH accumulation in the culture medium over a 30 min interval was evaluated by radioimmunoassay. Nicotine or its antagonists did not significantly affect basal GnRH release from GT1-1 cells (Fig. 2A). Similarly, exposure to the nAChR antagonists αBgtx and D-Tub did not significantly affect GnRH release.

3.3 Effects of the activation of nAChR by nicotine on pulsatile GnRH release

To explore whether the possible action of nicotine receptor activation on GnRH release could have modified the dynamic, rather than the total amount, of GnRH release, a series of perifusion experiments were performed.

GT1-1 cells were cultured as described in Materials and Method and perifused for 30 minutes with a 500 μM solution of nicotine. This concentration was selected considering the results obtained in the experiment on GnRH accumulation and the consolidated notion that nAch requires high concentrations of agonist to couple binding to channel opening and, conversely, low concentrations of agonist may induce a rapid desensitization of receptors followed by an increase

in their number (Quick and Lester, 2002); this phenomenon may produce biphasic responses that complicate biological interpretation. The medium was collected at different time intervals during the infusion and subjected to radioimmunoassay.

As expected, we found that the basal release of GnRH by GT1–1 cells is intrinsically pulsatile (Fig. 2B) with a mean pulse frequency of 2.58 ± 0.54 pulses/hour (mean ± SD), as detected by cluster analysis on 6 independent experiments (Magni et al., 1999). After a 30-60 min of preconditioning in basal conditions, nicotine (at 500 μM concentration) was added to the perifusion medium. The cells were exposed to nicotine for 30 min followed by a 30-40 min of washing The results show a change of the secretory peaks characterized by a significantly increased pulse frequency (5.13 ± 1.03 pulse/hour; $p<0.05$), associated with a reduced pulse duration during nicotine exposure and for few minutes after the withdrawal of treatment withdrawal, followed by a rapid return to a basal frequency (3.25 ± 0.35 pulse/hour)(Fig. 2B). The baseline secretory activity of GT1–1 cells, as well as their response to nicotine were different among perifusion experiments due to intrinsic features of this system; however, the effect of nicotine was qualitatively similar when measured over independent experiments. However, in agreement with static experiments, the amount of GnRH released, calculated as the area under the curve, was not significantly different before and during nicotine exposure (data not shown).

3.4 Effects of the activation of nAChR by nicotine on PGE_1 stimulated GnRH release and cAMP accumulation

Since the release of hypothalamic GnRH is regulated by several stimulatory inputs impinging on GnRH neurons (Hrabovszky and Liposits, 2013), we explored the possibility that the activation of nAChRs could exert an inhibitory effect on stimulated GnRH release.

The release of GnRH was induced by exposure of GT1-1 cells to PGE_1 as a GnRH secretagogue (Maggi et al., 1995; Pimpinelli et al., 1999) and treated with nicotine and the two nAChR antagonists □□T□□ and αBgtx.

Under these conditions, nicotine shows a significant and dose-dependent inhibition (IC_{50} 214.0 ± 26.6 μM) of GnRH accumulation induced by exposure of the cells to PGE_1 (1 mM) (Fig. 3A). The inhibitory effect of nicotine is completely reversed by the presence of either of the nicotinic general nAChR antagonist D-Tub or antagonist of homomeric nAChR αBgtx, which, when given alone, do not significantly affect GnRH release in either basal or stimulated conditions (Fig. 3B and 3C).

Since in GT1-1 cells the secretagogue effect of PGE_1 is mediated by intracellular accumulation of cAMP (Pimpinelli et al., 1999), we analyzed whether the effect of nicotine was mediated by modifications of the formation of this intracellular second messenger. The results (Figure 4) indicate that nicotine exposure does not affect the PGE_1-induced of cAMP accumulation in GT1-1 cells suggesting that a different intracellular pathway is involved.

3.5 Effects of nicotine on high K^+-induced GnRH release

The effect of nicotine under GnRH stimulation by direct GT1-1 cell depolarization was subsequently investigated. During direct cell depolarization, induced by the exposure to high extracellular K^+ concentration (56 mM) (Pimpinelli et al., 2003), nicotine still inhibits the release of GnRH in a dose-dependent manner (IC_{50} 121.7 ± 18.8 μM) (Fig. 5A); an effect that is reversed by the presence of either of the nicotine antagonists (Fig. 5B and 5C).

4. Discussion

At least two classes of functional nAChR receptors are expressed in GT1-1 immortalized GnRH neurons. One class of such receptors is bound by Epi, a potent cholinergic agonist (Houghtling et al., 1995), with high affinity (pM range) and specificity. Epi is known to interact with high affinity with many heteromeric nAch receptors, and in particular with α4β2 (Gotti et al., 1997), the most represented form in mouse brain and hypothalamus (Flores et al., 1992; Millar and Gotti, 2009). However, it may also bind homomeric α7 nAch receptors, although with an affinity that is four orders of magnitude lower (Sullivan et al., 1994).

Labeled αBgtx was also found to bind, with nM affinity, to a single class of sites on GT1-1 cell membranes. αBgtx selectively interacts with muscle nicotinic receptors (α1) and homomeric α7, expressed in different neural tissues, suggesting the presence of these receptors (McGehee and Role, 1995).

The presence of nAChR in GT1-1 cells was then confirmed by RT-PCR experiments that revealed the presence of transcripts specific for $\alpha 7$, $\alpha 4$ and $\beta 2$ nAChR subunits.

Considering the limitations of the *in vitro* cell model, these findings are in agreement with the reported main distribution of α7 and α4β2 nAChR in several hypothalamic structures and in the median eminence (Clarke et al., 1985; Dominguez del Toro et al., 1994; Flores et al., 1992; Michels et al., 1986; Millar and Gotti, 2009), the brain region where the axons of GnRH-secreting neurons make contact with the hypothalamo-pituitary portal vessels.

An anatomical relationship of cholinergic neuronal pathways and gonadotropin-releasing hormone neurons of the preoptic area has been indicated by the detection of cholinergic axons in apposition to gonadotropin-releasing hormone immunoreactive cell bodies and dendrites, providing direct neuromorphological evidence for the involvement of the cholinergic system in the regulation of gonadotropin-releasing hormone neurons (Turi et al., 2008). Similarly, we have previously demonstrated the presence of delta opiod receptors both on GT1-1 cells and in hypothalamic GnRH terminals (Maggi et al., 1995; Pimpinelli et al., 2006).

The data reported here indicate that the activation of nAChRs present in GT1-1 cells by nicotine does not modify the constitutive basal GnRH release, although increased pulse frequency and decreased pulse duration of GnRH secretion was observed on exposure to the alkaloid.

Using hypothalamic primary cell cultures and GT1-7 clone, Krsmanovic and coworkers (Krsmanovic et al., 1998) found a dual effect of Ach on GnRH neurons; while activation of M2 muscarinic receptors reduced basal GnRH release, the activation of M1 receptors resulted in a rapid and transient increase in GnRH neurosecretion. In addition, these authors reported that the treatment of GT1-7 neurons with nicotine (at 10 μM concentration) caused a transient increase in GnRH pulsatility, even if no further characterization of the nicotine receptor involved or its specific activation were provided.

On the other hand, the decrease of the pulse frequency reported here agrees with the observation that 'in vivo' nicotine may inhibit the activity of the GnRH pulse generator (Sano et al., 1999) by suppressing the neuronal multiunit activity (MUA) at the level of the median eminence. However, this study was carried out in ovariectomized animals, where the release of GnRH is highly stimulated, due to the lack of gonadal steroid-mediated negative feedback and the activation of neurostimulatory inputs. In fact, neurons in culture lack multiple neuronal afferents that impinge on hypothalamic GnRH neurons (Gore and Roberts, 1997; Pimpinelli et al., 1999). Accordingly, a potent dose-dependent inhibitory effect of nicotine was observed during the stimulation of GnRH release by PGE_1 or to high extracellular K^+ concentration, an experimental condition more similar to that of native hypothalamic GnRH neurons (Pimpinelli et al., 1999; Pimpinelli et al., 2003).

Both these two conditions are powerful stimuli to increase cytosolic Ca^{2+} levels (Krsmanovic et al., 1996; Martinez de la Escalera et al., 1995; Stojilkovic et al., 1994) that activates the release machinery of GnRH, even though they act with a different mechanism. In fact, while exposure to high K^+ induces a direct depolarization of the cell (Mellon et al., 1991; Pimpinelli et al., 2003), PGE_1 acts through the formation of cAMP (Pimpinelli et al., 1999). We have similarly reported that opioid peptides were unable to modify the basal secretion of GnRH from the GT1-1 cells but they exerted an inhibitory effect under stimulation of GnRH release with prostaglandins (PGE_1 and PGE_2) (Maggi et al., 1995).

This observation, indicating a significant inhibitory effect of nicotine on GnRH release, further affirms that GT1 cells resemble hypothalamic GnRH-secreting neurons only when they are properly stimulated (Gore and Roberts, 1997).

The inhibitory effect of nicotine, both on cells stimulated with K^+ or with PGE_1, was efficiently blocked by D-Tub confirming the interaction of nicotine with nAChR. Significantly, αBgtx also completely blocks the effect of nicotine, indicating the involvement of αBgtx-sensitive nAChR present in GT1-1 neurons (α7) in the control of secretion of GnRH.

Moreover, our investigation on the possible mechanism underlying these effects demonstrates that the inhibition of PGE_1 -induced secretion of GnRH by nicotine is not mediated by a change in cAMP accumulation, suggesting a possible action downstream to the activation of this intracellular pathway.

It has been described that the protein kinase A, protein kinase G and protein kinase C pathways are all functionally coupled to regulation of GnRH secretion by GT1 cells; in particular, the pulsatile secretion of GnRH is coupled to the entry of extracellular Ca^{2+} via L-type Ca^{2+} channels (Martinez de la Escalera et al., 1995; Zheng et al., 1997). Therefore cell depolarization triggers the Ca^{2+} entry response with consequent exocytosis of GnRH. The importance of cytoplasmic concentration of Ca^{2+} in GnRH release has been established both in native GnRH (Drouva et al., 1981; Ojeda et al., 1988) and GT1 neurons (Krsmanovic et al., 1992).

Prostaglandins were found to induces a membrane depolarization in native mouse GnRH neurons by the involvement of a non-selective cation current that require the cAMP/protein kinase A (PKA) pathway activation (Clasadonte et al., 2011; Coleman et al., 1994; Ojeda and Negro-Vilar, 1985; Roland and Moenter, 2011; Sang et al., 2005; Zhang et al., 2008). Accordingly, prostaglandin induces GnRH release in GT1 cells by promotion of cAMP formation and calcium mobilization (Ojeda and Negro-Vilar, 1985; Ojeda et al., 1985; Rage et al., 1997)

Direct membrane depolarization induced by high extracellular concentration of K^+ (from 7.5 to 60 mM) also induces a dose-dependent increase of intracellular Ca^{2+} levels in GT1 neurons, with a consequent GnRH release, that involves voltage dependent N- and L-type Ca^{2+} channels (Javors et al., 1995; Krsmanovic et al., 1992; Stojilkovic et al., 1994). The action of K^+ on GnRH release is not affected by a pretreatment with TTX excluding the involvement of sodium channels in such effect (Mellon et al., 1991).

Collectively, several studies confirm that depolarization of the plasma membrane and influx of Ca^{2+} through L-type, and possibly N-type, calcium channels are associated functionally with the stimulated release of GnRH and may be shared by PGE_1 and K^+ action. Therefore, the observation that nicotine inhibits the PGE_1-induced GnRH release from GT1-1 cells without affecting the cAMP formation as well as blocks the release of GnRH promoted by exposure to high extracellular concentration of K^+, lead to hypothesize that the activation of nAchRs might affect membrane depolarization. According to their pharmacological profile, the activation nAChRs may change ion permeability of the GT1-1 cell membrane, making the cells insensitive to external stimuli irrespective of the intracellular pathway activated. A postsynaptic inhibitory action of nAChRs in central neurons is supported by a study carried out on rat brain slices, in which the application of

nicotine was found to induce a marked neuronal hyperpolarization (Wong and Gallagher, 1989); however, the analysis of membrane ions currents under nicotine exposure by targeted electrophysiological experiments will help to clarify such hypothesis.

It has been proposed that the inhibitory action of nicotine observed *in vivo* is not due to a direct effect of the cholinergic agonist on GnRH neurons. The initial hypothesis suggesting the involvement of the opioid system (Pomerleau, 1998) was excluded by the observed insensitivity of nicotine effect to the opioid antagonist naloxone (Sano et al., 1999); more recently, it has been proposed that nicotine may stimulate the release of GABA which may inhibits GnRH release through GABA-A receptor (Kimura et al., 2004).

It should be underlined that the results from experiments in organotypic cultures or *in vivo* may be altered by the interference of other neuronal inputs to GnRH neurons possibly activated or inhibited by modification of the cholinergic tone, possibly by pharmacological doses of nicotine that might not reflect the physiological role of the activation of nAChRs.

GT1-1 cells may release GABA (Ahnert-Hilger et al., 1998) and the activation of GABA-A receptors induces an initial stimulatory action on GnRH release followed by an inhibitory phase possibly mediated by GABA-B receptors (Martinez de la Escalera et al., 1994). However, in contrast with the present results, in this case the inhibitory effect is mediated by a decrease of intracellular cAMP levels (Martin et al., 2007).

Results from GT1-1 cells seem to confirm the hypothesis that the effects of nicotine on the reproductive system "in vivo" can be largely mediated by a direct inhibitory action on GnRH neurons, mediated by αBgtx-sensitive receptors (α7 nAChR); this result does not exclude that 'in vivo' an additional indirect action of nicotine might be mediated by the activation of opioidergic or GABA interneurons.

Our results apparently contrast with a previous study (Krsmanovic et al., 1998) suggesting that the activation of nicotinic receptors in perifused immortalized GnRH neurons causes a prompt transient increase in basal GnRH release followed by a return to basal levels.

The discrepancy might be due to the different experimental procedures adopted in the two studies. Krsmanovic and coworkers (Krsmanovic et al., 1998) used immortalized GnRH neurons exposed to a lower concentration of nicotine (10 μM), than in the present perifusion study (500 μM), and the effect of nicotine was not tested in conditions of stimulated GnRH release.

Moreover, although the nAChR involved in the observed phenomena was not characterized, it is possible to speculate that exposure of the cells to low concentrations of nicotine might have induced a receptor desensitization-upregulation and biphasic response (as stated in the Results section) (Quick and Lester, 2002).

The results of our study indicate that the GnRH release-inhibitory potency of nicotine (IC_{50} 100-200 μM) is congruent with the potency found in activating channel current in neuronal cells (Arneric et al., 1994) and its affinity to rat brain α7 nAChR (Rueter et al., 2006).

Evidence of a direct inhibitory effect of nicotine in central neurons, provided by studies in which nicotine application reduced Purkinje cells discharge (de la Garza et al., 1989) or induced membrane hyperpolarization mediated by an increase in potassium conductance (Wong and Gallagher, 1989), are consistent with the results of the current study.

It is interesting to note that a greater number of nicotinic receptors are present in the brain of smokers (Benwell et al., 1988) since chronic nicotine induces a receptor upregulation with a partial receptor desensitization (Govind et al., 2009); this is true for α4/β2 nAChRs, the main high-affinity nicotine binding sites present in the brain; however, α7 nAChR were found not to be inactivated after up-regulation induced by chronic nicotine exposure (Kawai and Berg, 2001) making smokers more prone to the inhibitory effects of nicotine on GnRH release.

In conclusion, the results presented in this study demonstrate for the first time the presence of at least two classes of nAChRs on immortalized GnRH neurons (GT1-1 cell line) and that the activation of the αBgtx-sensitive subclass (possibly α7) produces an inhibitory effect on the release of GnRH.

Acknowledgements

This work was partially supported by the Italian National Research Council (CNR), by the Italian Ministry for Universities and Research (MIUR) and from Università degli Studi di Milano (Research Support Plan, line 2, action B 2017-18). We thank Dr. David S. Horner (Dept. of Bioscience, University of Milano) for the language editing of the manuscript.

Conflict of interest

The authors declare no competing or financial interests.

Author contributions

E.M., F.P., C.G and R.M. conceived the project.
E.M., F.P. and R.M. performed all the experiments on living cells
C.G. performed binding experiments.
V.A. performed RT-PCR experiments
C.G., V.A., C.R. and R.M. assisted with data interpretation and manuscript preparation.

Legends to figures

Figure 1 Detection of nicotinic acetylcholine receptors in GT1-1 cells.

Homologous saturation curves of the binding of (A) ^{3}H-epibatidine (^{3}H-Epi) and (B) ^{125}I-a-bungarotoxin (^{125}I-αBgtx) on GT1-1 (circle) and HEK392 (triangle) cell membrane preparations Binding isotherms have been analyzed by LIGAND program. (C) RT-PCR amplified transcripts for α4, α7 and β2 nicotine receptor subunits in GT1-1 cell total RNA. Normal adult mouse brain total RNA was used as internal control. Mouse *Gapdh* was used as housekeeping gene.

Figure 2 Effect of the exposure to nicotine on the basal release of GnRH from GT1-1 cells.

(A) Basal accumulation of immunoreactive GnRH in culture medium of GT1-1 cells after a 30 min static incubation in control (C) or in presence of increasing concentration of nicotine and cholinergic antagonists αBgtx (2.5 mM) and D-Tub (250 mM). Values are expressed as mean ± SEM (n=8). (B) Representative graphs (of four separate experiments) of perifused GT1-1 cells in absence and in presence of a 500 μM concentration of nicotine; the segmented bar on top of the profile of GnRH release indicates the position and duration of secretory pulses identified by cluster analysis.

Figure 3 Effect of the exposure to nicotine on the release of GnRH induced by PGE_1 .

(A) Representative dose response curve of the inhibitory action of nicotine on PGE_1-induced GnRH release. The effect of nicotine is abolished in the presence of the antagonists (B) D-Tub and (C) αBgtx. Values are expressed as mean ± SEM (n=8); § $p<0.05$ vs basal release levels (B); * $p<0.05$ vs 0.

Figure 4 Nicotine does not modify the PGE_1-induced cAMP accumulation in GT1-1 cells.

The exposure of GT1-1 cells to nicotine or the antagonist D-Tub does not affect the intracellular accumulation of cAMP induced by PGE_1.
Values are expressed as mean ± SEM (n=8); § $p<0.05$ vs basal release levels (B).

Figure 5 Effect of the exposure to nicotine on the release of GnRH induced by 56 mM K^+- .
(A) Representative dose response curve of the inhibitory action of nicotine on 56 mM K^+-induced GnRH release. The effect of nicotine is abolished in the presence of the antagonists (B) D-Tub and (C) αBgtx. Values are expressed as mean ± SEM (n=8); § $p<0.05$ vs basal release levels (B); * $p<0.05$ vs 0.

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Highlights

- Immortalized GnRH neurons (GT1-1) express acetylcholine nicotinic receptors
- Nicotine has not effect on basal accumulation of GnRH
- Nicotine affects the pulsatility of GnRH release from GT1-1 cells
- Nicotine exerts an inhibitory action on stimulated GnRH release
- The effect of nicotine does not affect intracellular cAMP levels

Figure1

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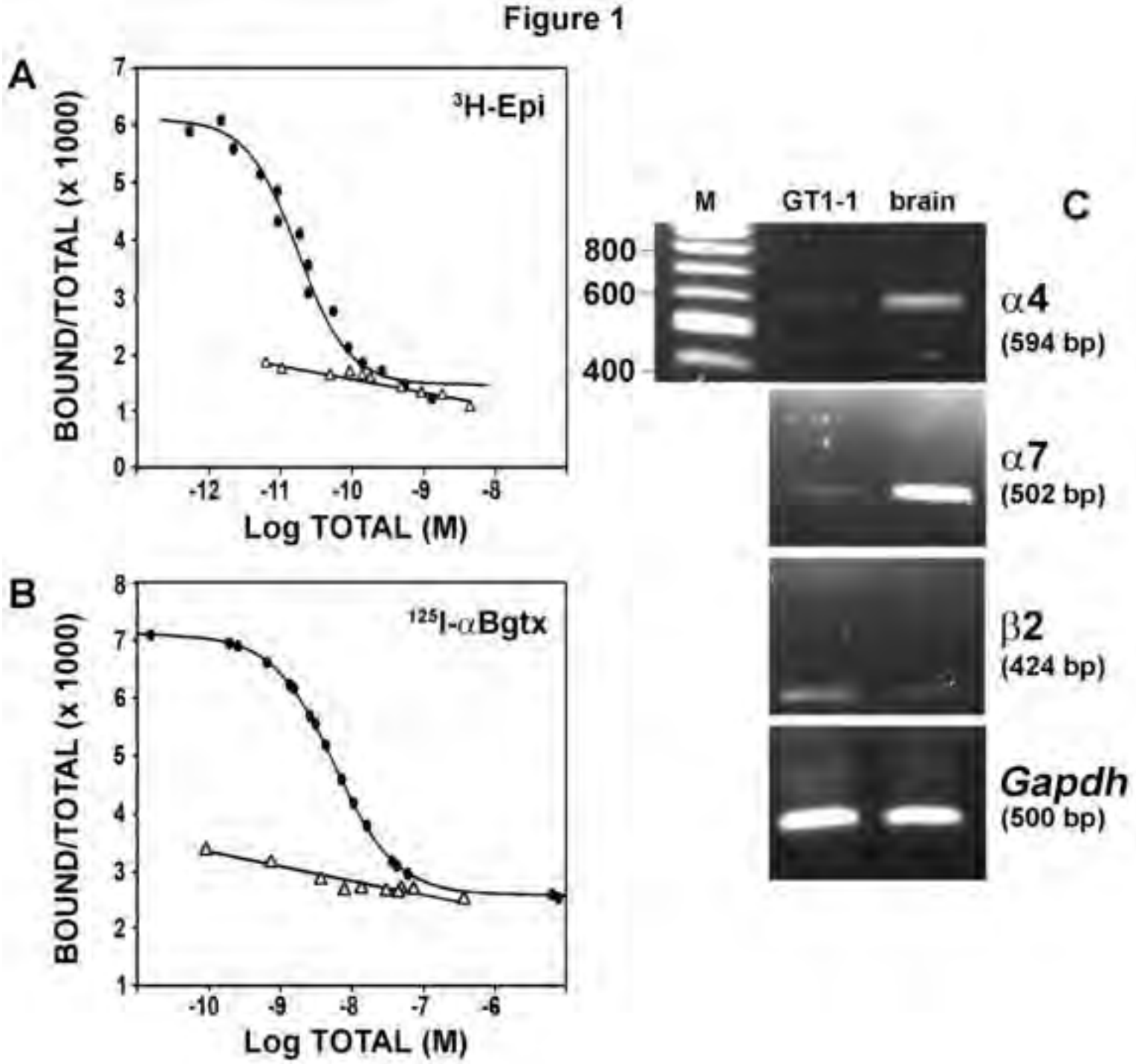

Figure2

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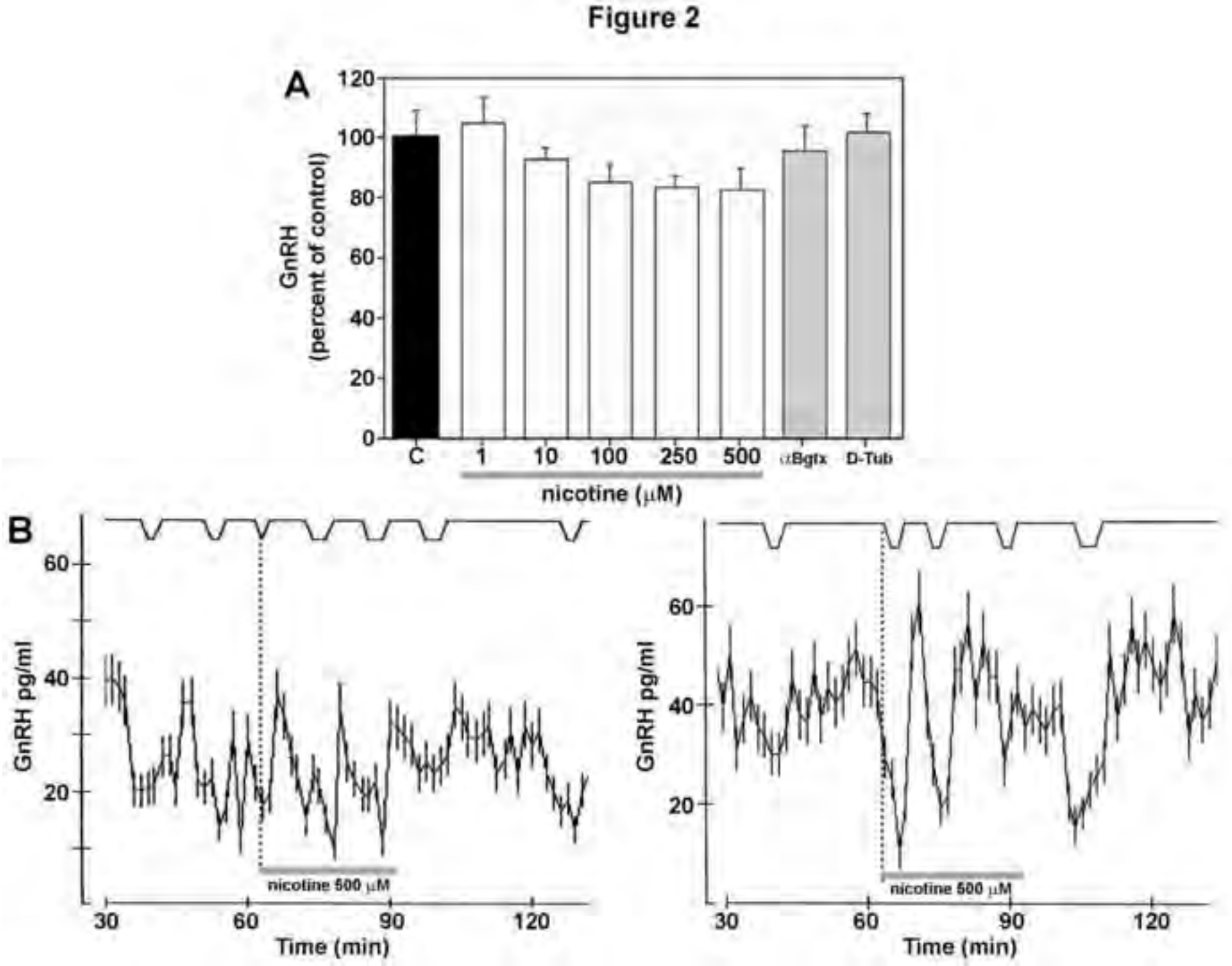

Figure3

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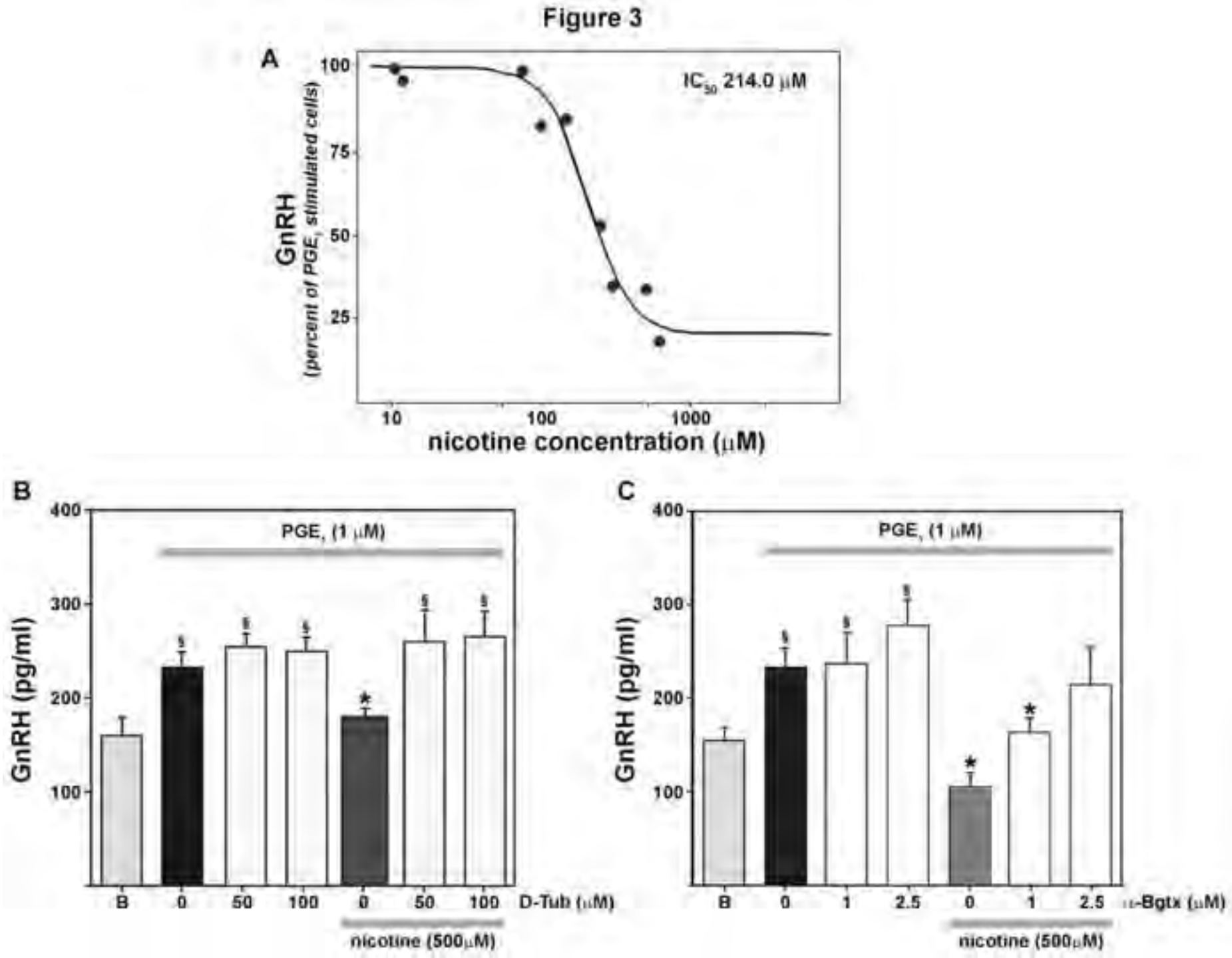

Figure4

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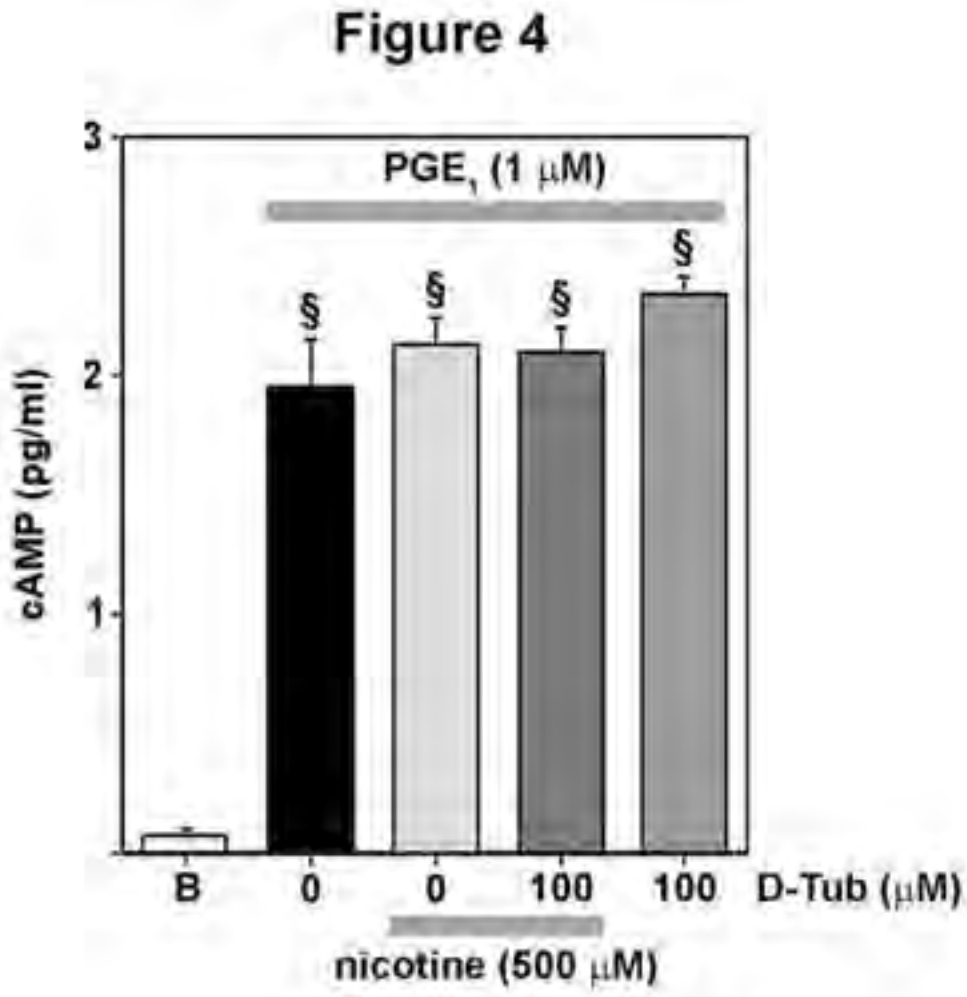

Figure5

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